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GB0317466.1

By virtue of a direction given under Section 30 of the Patents Act 1977, the application is proceeding in the name of

THE UNIVERSITY OF SHEFFIELD,
Incorporated in the United Kingdom,
Western Bank,
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[ADP No. 00773812002]

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3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — THE UNIVERSITY OF SHEFFIELD, Western Bank, SHEFFIELD, S10 2TN 77381200 2

THE UNIVERSITY OF NEWCASTLE, 6 Kensington Terrace, NEWCASTLE-UPON-TYNE, NE1 7RU 6179166 2001

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SECTION 34(1) APPLICATION

4. Title of the invention — Use

5. Name of your agent *(if you have one)* — Harrison Goddard Foote

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)* — 31 St Saviourgate
YORK
YO1 8NQ

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11. I/We request the grant of a patent on the basis of this application.

Signature: Harrison Goddard Foote — Date: 25 July 2003

12. Name and daytime telephone number of person to contact in the United Kingdom: Michelle O'Neill — 01904 732120

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USE

This invention relates to the use of an agent that inhibits the activity of an enzyme which mediates repair of single strand breaks in DNA in the treatment of certain forms of cancer in particular breast cancer.

Homologous recombination (HR) has been shown to play an important role in repair of damage occurring at DNA replication forks in mammalian cells (2). Thus, cells deficient in HR show retarded growth and exhibit higher level of genetic instability. It is believed that genetic instability due to loss of HR repair in human cancers significantly contributes to the development of cancer in these cells (1).

Post-transcriptional modification of nuclear proteins by poly(ADP-ribosyl)ation (PARP) in response to DNA strand breaks plays an important role in DNA repair, regulation of apoptosis, and maintenance of genomic stability.

Poly(ADP-ribose) Polymerase (PARP-1) is an abundant nuclear protein in mammalian cells that catalyses the formation of poly(ADP-ribose) (PAR) polymers using NAD^+ as substrate. Upon DNA damage, PARP-1 binds rapidly to a DNA single-strand break (SSB) and catalyses the addition of negatively charged PAR chains to itself (automodification) and other proteins (see (3, 4) for reviews). The binding of PARP-1 to SSBs is believed to protect DNA lesions from further processing until PARP-1 is dissociated from the break by the accumulated negative charge resulting from PAR polymers (5,6).

Although PARP-1 has been implicated in several nuclear processes, such as modulation of chromatin structure, DNA replication, DNA repair and transcription, PARP-1 knockout mice develop normally (7). Cells isolated from these mice exhibit a hyper recombination phenotype and genetic instability in the form of increased levels of SCE, micronuclei and tetraploidy (8-10). Genetic instability may also occur in these PARP-1 knockout mice through telomere shortening, increased frequency of chromosome fusion and aneuploidy (11), although all of these results could not be repeated in another set of PARP-1 knock-out mice (12). In the former mice knockout, PARP-1 null mutation rescue impaired V(D)J recombination in SCID mice (13).

These results support the view suggested by Lindahl and coworkers that PARP-1 has a protective role against recombination (5). They proposed that binding of PARP-1 to ssDNA breaks prevents the recombination machinery from recognizing and processing DNA lesions or, alternatively, that the negative charges accumulated following poly ADP-ribosylation repel adjacent recombinogenic DNA sequences. Only the latter model is consistent with inhibition of PARP-1 itself and expression of a dominant negative mutant PARP-1, inducing SCE, gene amplification and homologous recombination (HR [14-18]).

Studies based on treating cells with inhibitors of PARP-1 or cells derived from PARP-1 knockout mice indicate that the suppression of PARP-1 activity increases cell susceptibility to DNA damaging agents and inhibits strand break rejoining (3, 4, 8-11, 19, 20).

Inhibitors of PARP-1 activity have been used in combination with traditional anti-cancer agents such as radio therapy and chemotherapy (21). The inhibitors were used in combination with methylating agents, topoisomerase poisons and ionising radiations and were found to enhance the effectiveness of these forms of treatment. Such treatments, however, are known to cause damage and death to non cancerous or "healthy" cells and are associated with unpleasant side effects.

There is therefore a need for a treatment for cancer that is both effective and selective in the killing of cancer cells and which does not need to be administered in combination with radio or chemotherapy treatments.

The present inventors have surprisingly found that cells deficient in homologous recombination (HR) are hypersensitive to inhibitors of PARP-1 as compared to wild type cells. This is surprising since PARP-1 knockout mice live normally.

According to a first aspect of the invention there is provided the use of an agent which inhibits the activity of an enzyme which mediates repair of single strand breaks in DNA in the manufacture of a medicament for the treatment of diseases/conditions which are caused by a genetic defect in a gene which mediates homologous recombination.

In a further aspect the invention provides a method of treatment of a disease or condition in a mammal, including human, which is caused by a genetic defect in a gene which mediates homologous recombination, which method comprises administering to the mammal a therapeutically effective amount of an agent which inhibits the activity of an enzyme which mediates repair of single strand breaks in DNA.

In a preferred aspect said enzyme is PARP. In a further preferred aspect said agent is a PARP inhibitor or an RNAi molecule specific to PARP gene.

In a further preferred aspect, the use is in the treatment of cancer wherein the cancer is caused by a genetic defect in a gene which mediates homologous recombination.

Preferably the medicament is a pharmaceutical composition consisting of the PARP inhibitor in combination with a pharmaceutically acceptable carrier or diluent.

The specific sensitivity of HR tumours to PARP-1 inhibition means that normally dividing cells in the patient will be unaffected by the treatment. Treatment of HR defective cancer cells using a PARP inhibitor also has the advantage that it does not need to be administered as a combination therapy along with conventional radio or chemotherapy treatments thereby avoiding the side effects associated with these conventional forms of treatment.

A genetic defect in a gene which mediates homologous recombination may be due to a mutation in, the absence of, or defective expression of, a gene encoding a protein involved in HR.

In a further aspect, the invention further provides the use of a PARP inhibitor in the manufacture of a medicament for inducing apoptosis in HR defective cells.

In another aspect the invention provides a method of inducing apoptosis in HR defective cells in a mammal which method comprises administering to the mammal a therapeutically effective amount of a PARP inhibitor. By causing apoptosis in HR

defective cells it should be possible to reduce or halt the growth of a tumour in the mammal.

Preferably, the HR defective cells are cancer cells.

Cancer cells defective in HR may partially or totally deficient in HR. Preferably the cancer cells are totally deficient in HR.

The term "cancer" or "tumour" includes cancer of the lung, colon, pancreatic, gastric, ovarian, cervical, breast or prostate cancer. In a preferred aspect, the cancer is in a mammal, preferably human.

The cancer to be treated may be an inherited form of cancer wherein the patient to be treated has a familial predisposition to the cancer. Preferably, the cancer to be treated is gene-linked hereditary cancer. In a preferred embodiment of the invention the cancer is gene-linked hereditary breast cancer.

In a preferred aspect, the PARP inhibitor is useful in the treatment of cancer cells defective in the expression of a gene involved in HR. Genes with suggested function in HR include XRCC1, ADPRT (PARP-1), ADPRTL2 (PARP-2), CTPS, RPA, RPA1, RPA2, RPA3, XPD, ERCC1, XPF, MMS19, RAD51; RAD51B, RAD51C, RAD51D, DMC1, XRCC2, XRCC3, BRCA1, BRCA2, RAD52, RAD54, RAD50, MRE11, NBS1, WRN, BLM, Ku70, Ku80, ATM, ATR, chk1, chk2, FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE, FANCF, FANCG, RAD1, RAD9 (see (2, 3, 5, 22-28) for reviews).

A gene involved in HR may be a tumour suppressor gene. The invention thus provides for the treatment of cancer cells defective in the expression of a tumour suppressor gene. Preferably, the tumour suppressor gene is BRCA1 or BRCA2.

Breast cancer is the most common cancer disease among women in the Western world today. Certain families have strong predisposition for breast cancer, which is often owing to an inherited mutation in one allele of either BRCA1 or BRCA2. However, these patients still maintain one functional allele. Thus, these patient develop normally

and have no phenotypic consequence from this mutation. However, in one cell, the functional allele might be lost, making this cell cancerous and at the same time deficient in homologous recombination (HR). This step is critical for the onset of a tumour [1].

The present inventors have surprisingly found that BRCA2 deficient cells are 100 times more sensitive to the PARP-1 inhibitor, NU1025, than wild type cells.

Thus in a preferred aspect, the invention provides the use of a PARP inhibitor in the manufacture of a medicament for the treatment of cancer cells defective in BRCA1 and/or BRCA2 expression.

The cancer cells to be treated may be partially or totally deficient in BRCA1 or BRCA2 expression. BRCA1 and BRCA2 mutations can be identified using multiplex PCR techniques, array techniques (29, 30) or using other screens known to the skilled person.

PARP inhibitors useful in the present invention may be selected from inhibitors of PARP-1, PARP-2, PARP-3, PARP-4, tankyrase 1 or tankyrase 2 (see 31 for a review). In a preferred embodiment, the PARP inhibitor useful in the present invention is an inhibitor of PARP-1 activity.

PARP inhibitors useful in the present invention include benzimidazole-carboxamides, quinazolin-4-[3H]-ones and isoquinoline derivatives (e.g.2-(4-hydroxyphenyl)benzimidazole-4-carboxamide (NU1085), 8-hydroxy-2-methylquinazolin-4-[3H]one (NU1025); 6(5H)phenanthridinone; 3 aminobenzamide; AG14361; benzimidazole-4-carboxamides (BZ1-6) and tricyclic lactam indoles (TI1-5) [32]. Further inhibitors of PARP may be identified either by design [33] or the novel FlashPlate assay [34].

The PARP inhibitor formulated as a pharmaceutical composition may be administered in any effective, convenient manner effective for targeting cancer cells including, for instance, administration by oral, intravenous, intramuscular, intradermal, intranasal, topical routes among others. Carriers or diluents useful in the pharmaceutical

composition may include, but are not limited to saline, buffered saline, dextrose, water, glycerol, ethanol and combinations thereof.

In therapy or as a prophylactic, the active agent may be administered to an individual as an injectable composition, for example as a sterile aqueous dispersion. The inhibitor may be administered directly to a tumour or may be targeted to the tumour via systemic administration.

A therapeutically effective amount of the inhibitor is typically one which is sufficient to achieve the desired effect and may vary according to the nature and severity of the disease condition, and the potency of the inhibitor. It will be appreciated that different concentrations may be employed for prophylaxis than for treatment of an active disease.

For administration to mammals, and particularly humans, it is expected that the daily dosage level of the active agent will be from 0.01mg/kg to 10 mg/kg body weight, typically up to 0.1, 05, 1.0, 2.0 or 5.0 mg/kg body weight. Ultimately, however, the amount of inhibitor administered and the frequency of administration will be at the discretion of a physician.

A therapeutic advantage of using PARP inhibitors to treat cancer cells is that only very low doses are needed to have a therapeutic effect in treating cancer thereby reducing systemic build up of the inhibitors and any associated toxic effects.

A preferred aspect of the invention provides an agent which is an inhibitory RNA (RNAi) molecule.

A technique to specifically ablate gene function is through the introduction of double stranded RNA, also referred to as inhibitory RNA (RNAi), into a cell which results in the destruction of mRNA complementary to the sequence included in the RNAi molecule. The RNAi molecule comprises two complementary strands of RNA (a sense strand and an antisense strand) annealed to each other to form a double stranded RNA molecule. The RNAi molecule is typically derived from exonic or coding sequence of the gene which is to be ablated.

Preferably said RNAi molecule is derived from the nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of:

a) a nucleic acid sequence as represented by the sequence in Figure 5, 6, 7, 8, 9 or 10, or fragment thereof;

b) a nucleic acid sequence which hybridises to the nucleic acid sequences of Figure 5, 6, 7, 8, 9 or 10 and encodes a gene for PARP;

c) a nucleic acid sequence which comprise sequences which are degenerate as a result of the genetic code to the nucleic acid sequences defined in (a) and (b).

Recent studies suggest that RNAi molecules ranging from 100-1000bp derived from coding sequence are effective inhibitors of gene expression. Surprisingly, only a few molecules of RNAi are required to block gene expression which implies the mechanism is catalytic. The site of action appears to be nuclear as little if any RNAi is detectable in the cytoplasm of cells indicating that RNAi exerts its effect during mRNA synthesis or processing.

The exact mechanism of RNAi action is unknown although there are theories to explain this phenomenon. For example, all organisms have evolved protective mechanisms to limit the effects of exogenous gene expression. For example, a virus often causes deleterious effects on the organism it infects. Viral gene expression and/or replication therefore needs to be repressed. In addition, the rapid development of genetic transformation and the provision of transgenic plants and animals has led to the realisation that transgenes are also recognised as foreign nucleic acid and subjected to phenomena variously called quelling (Singer and Selker, 1995), gene silencing (Matzke and Matzke, 1998) , and co-suppression (Stam *et. al.*, 2000).

Initial studies using RNAi used the nematode *Caenorhabditis elegans*. RNAi injected into the worm resulted in the disappearance of polypeptides corresponding to the gene sequences comprising the RNAi molecule(Montgomery et. al., 1998; Fire et. al., 1998). More recently the phenomenon of RNAi inhibition has been shown in a number of eukaryotes including, by example and not by way of limitation, plants, trypanosomes (Shi et. al., 2000) *Drosophila spp.* (Kennerdell and Carthew, 2000).

Recent experiments have shown that RNAi may also function in higher eukaryotes. For example, it has been shown that RNAi can ablate *c-mos* in a mouse ooctye and also E-cadherin in a mouse preimplanation embryo (Wianny and Zernicka-Goetz, 2000).

More preferably said RNAi molecule according has a length of between 10 nucleotide bases (nb) –1000nb. Even more preferably said RNAi molecule has a length of 10nb; 20nb; 30nb; 40nb; 50nb; 60nb; 70nb; 80nb; 90nb; or 100bp. Even more preferably still said RNAi molecule is 21nb in length.

Even more preferably still the RNAi molecule comprises the nucleic acid sequence aaa agc cau ggu gga gua uga (PARP-1)

Even more preferably still the RNAi molecule consists of the nucleic acid sequence aag acc aau cuc ucc agu uca ac (PARP-2)

Even more preferably still the RNAi molecule consists of the nucleic acid sequence aag acc aac auc gag aac aac (PARP-3)

The RNAi molecule may comprise modified nucleotide bases.

Preferred features of each aspect of the invention are as for each of the other aspects *mutatis mutandis*.

The present invention will now be described by way of example only with reference to the accompanying figures, wherein:

Figure 1 is a graph demonstrating that HR deficient cells are hypersensitive to the toxic effect caused by inhibition of PARP-1. Colony outgrowth of the Chinese hamster cell lines AA8 (wild-type), irs1SF (deficient in HR[4]), CXR3 (irs1SF complemented with XRCC3 [2]), V79 (wild-type), irs1 (deficient in HR[5]) or irs1X2.2 (irs1 complimented with XRCC2 [1]) upon exposure to 3-AB (A), ISQ (B) or NU1025 (C). The means (symbols) and standard deviation (bars) of at least three experiments are shown. Colony outgrowth assay was used;

Figure 2 is a graph showing cell survival in the presence of PARP inhibitor NU1025 in wt V79 cells, BRCA2 deficient VC-8 cells and VC-8 cells complimented with functional BRCA2 gene (VC-8#13, VC-8+B2). Colony outgrowth assay was used;

Figure 3 is a graph showing cell survival in the presence of PARP inhibitor AG14361 in wt V79 cells, BRCA2 deficient VC-8 cells and VC-8 cells complimented with functional BRCA2 gene (VC-8#13, VC-8+B2);

Figure 4 is a histogram showing the percentage of the cells in apoptosis following a 72 hour incubation with NU1025;

Figure 5 is the human cDNA sequence of PARP-1;

Figure 6 is the human cDNA sequence of PARP-2;

Figure 7 is the human cDNA sequnce of PARP-3;

Figure 8 is the human gDNA sequnce of Tankyrase 1;

Figure 9 is the human mRNA sequnce of Tankyrase 2;

Figure 10 is the human mRNA sequnce of VPARP;

EXAMPLES

Homologous recombination deficient cells are hypersensitive to PARP-1 inhibition

To investigate the involvement of HR in cellular responses to inhibition of PARP-1, the effects of PARP-1 inhibitors on the survival of HR repair deficient cell lines were studied. It was found that cells deficient in HR (i.e., irs1SF which is defective in XRCC3 or irs1 which is defective in XRCC2 [see Table 1] were very sensitive to the toxic effect of 3-aminobenzamide (3-AB) and to two more potent inhibitors of PARP-1: 1,5-dihydroxyisoquinoline (ISQ; [37]) or 8-hydroxy-2-methylquinazolinone (NU1025 [38, 39]) (Figure 1). The sensitivity in irs1SF cells to 3-AB, ISQ or NU1025 was corrected by the introduction of a cosmid containing a functional *XRCC3* gene (CXR3). Similarly, the sensitivity in irs1 cells to 3-AB, ISQ or NU1025 was corrected by the introduction of a cosmid containing a functional *XRCC2* gene (irs1X2.2).

BRCA2 deficient cells are hypersensitive to PARP-1 inhibition

The survival of BRCA2 deficient cells (VC8) and wild type cells (V79Z) in the presence of inhibitors of PARP-1 was investigated. It was found that VC8 cells are very sensitive to the toxic effect of NU1025 (Figure 2) or AG14361 (Figure 3). The sensitivity in VC8 cells was corrected by the introduction of a functional BRCA2 gene either on chromosome 13 (VC8#13) or on an overexpression vector (VC8+B2). This result demonstrates that the sensitivity to PARP-1 inhibitors is a direct consequence of loss of the BRCA2 function.

To investigate if inhibition of PARP-1 triggers apoptosis in BRCA2 deficient cells, the level of apoptosis 72 hours following exposure to NU1025 was investigated. It was found that NU1025 triggered apoptosis only in VC8 cells, showing that loss of PARP-1 activity in BRCA2 deficient cells triggers this means of death (Figure 4).

Table 1. Genotype and origin of cell lines used in this study.

Cell line	Genotype	Defect	Origin	Reference
AA8	wt	wt	CHO	[41]
irs1SF	*XRCC3*$^-$	XRCC3$^-$, deficient in HR	AA8	[41]
CXR3	*XRCC3*$^-$	wt	irs1SF	[41]
	+ *hXRCC3*			
V79-4	wt	wt	V79	[40]
irs1	*XRCC2*$^-$	XRCC2$^-$, deficient in HR	V79-4	[40]
irs1X2.2	*XRCC2*$^-$	wt	irs1	[40]
	+ *hXRCC2*			
V79-Z	*wt*	wt	V79	[42]
VC8	*BRCA2*$^-$	BRCA2$^-$, deficient in HR	V79-Z	[42]
VC8#13	*BRCA2*$^-$	wt	VC8	[42]
	+hBRCA2			
VC8+B2	*BRCA2*$^-$	wt	VC8	[42]
	+hBRCA2			

Materials and Methods

Cytotoxicity of BRCA2 cells to PARP inhibitors

Cell culture

The irs1, irs1X2.1 and V79-4 cell lines were a donation from John Thacker [40] and the AA8, irs1SF and CXR3 cell lines were provided by Larry Thompson [41].

The VC-8, VC-8+B2, VC-8#13 were a gift from Malgorzata Zdzienicka [42]. All cell lines in this study were grown in Dulbecco's modified Eagle's Medium (DMEM) with 10% Foetalbovine serum and penicillin (100 U/ml) and streptomycin sulphate (100 µg/mL) at 37°C under an atmosphere containing 5% CO_2.

Toxicity assay - colony outgrowth assay

500 cells suspended in medium were plated onto a Petri dish 4 hours prior to the addition of 3-AB, ISQ or NU1025. ISQ and NU1025 were dissolved in DMSO to a final concentration of 0.2% in treatment medium. 7 - 12 days later, when colonies could be observed, these colonies were fixed and stained with methylene blue in methanol (4 g/l). Colonies consisting of more than 50 cells were subsequently counted.

Toxicity assay - clonogenic survival assay

Exponentially growing cells in 6-well plates were exposed to AG14361 in 1% DMSO or 1% DMSO alone in medium for 24 hours.

The cells were harvested by trypsinisation, counted and seeded at varying densities in 10 cm dishes in fresh medium in the absence of drug for colony formation.

7-10 days later the dishes were fixed with methanol:acetic acid 3:1 and stained with 0.4% crystal violet.

Colonies were counted and the survival relative to 1%DMSO control treated cells calculated.

Apoptosis experiments

$0.25x10^6$ cells were plated onto Petri dishes and grown for 4 hours before treatment with NU1025. After 72 hours, cells were trypsinized and resuspended with medium containing any floating cells from that sample. The cells were pelleted by centrifugation and resuspended for apoptosis analysis with FITC-conjugated annexin-V and propidium iodine (PI) (ApoTarget, Biosource International) according to manufacturer's protocol. Samples were analysed by flow cytometry (Becton-Dickenson FACSort , 488 nm laser), and percentage of apoptotic cells was determined by the fraction of live cells (PI-negative) bound with FITC-conjugated annexin-V.

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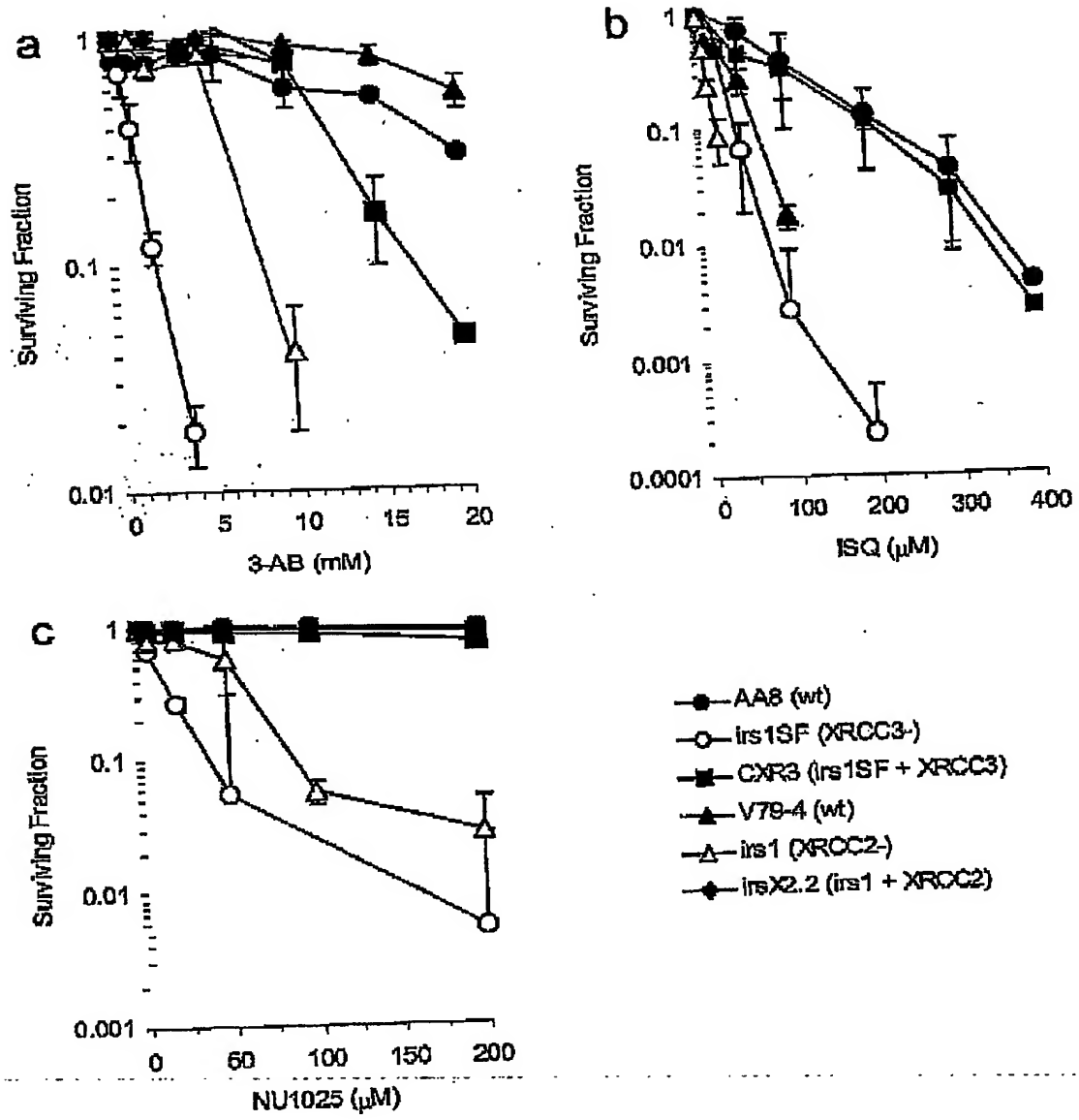
1/13

Figure 1.



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2/13

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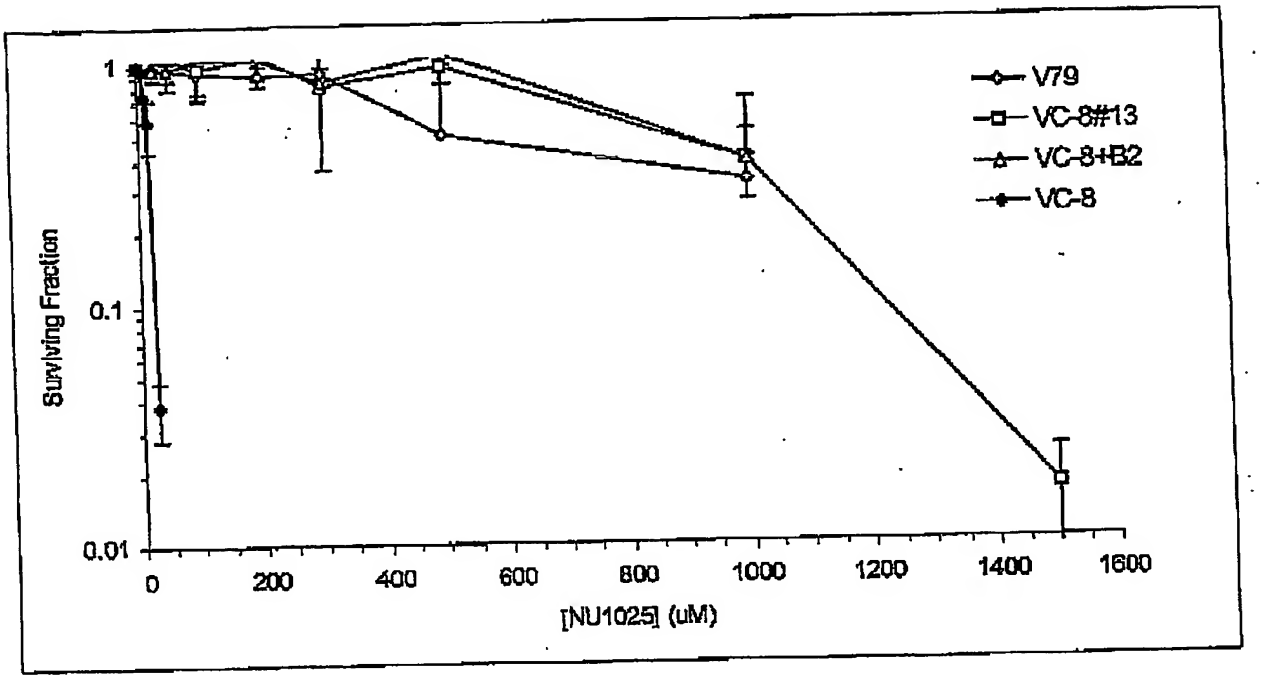

3/13

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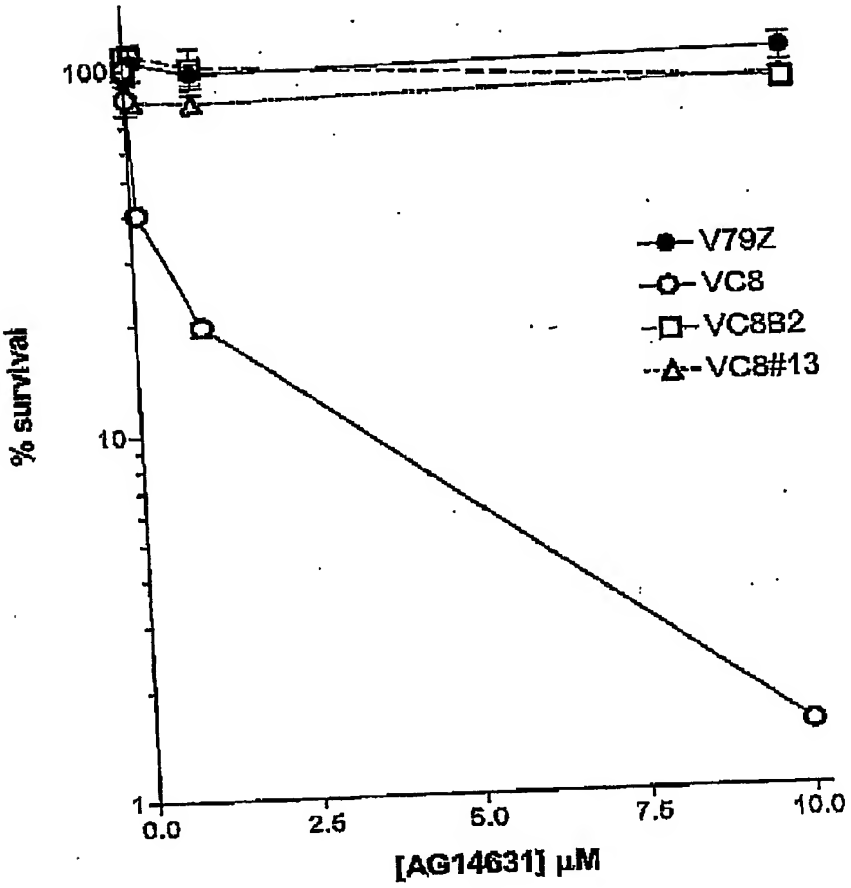

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4/13

Figure 4

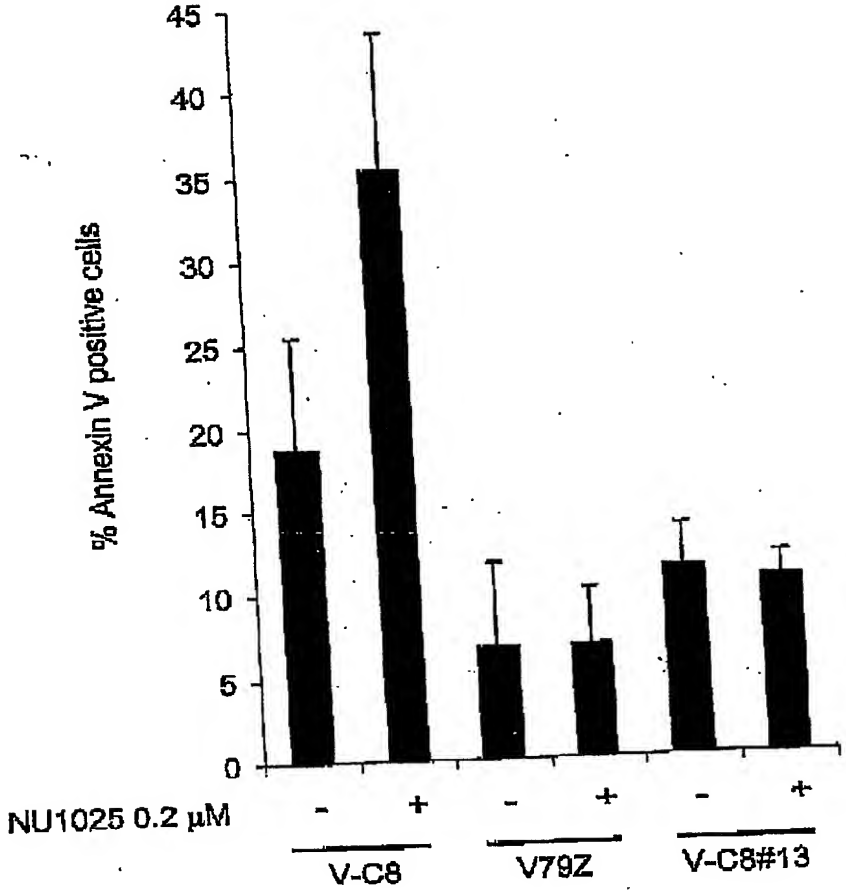

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FIGURE 5

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6/13

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7113

Figure 6

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Figure 7

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Figure 8

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Figure 9

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Figure 10

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